Role of insulin therapy in the gestational diabetes mellitus-associated hENT1 reduction in the fetoplacental vasculature endothelium

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Abstract

Gestational diabetes mellitus (GDM) is characterized by maternal glucose intolerance and hyperglycemia. The main goal of GDM treatment is to reduce the maternal hyperglycemia. For this, the first line of treatment is caloric and carbohydrates-restricted diet (GDMd). However, in cases in which mothers remain unresponsive to diet, insulin therapy (GDMi) is recommended. In the fetoplacental vasculature, GDM is associated with endothelial dysfunction. One of the main characteristics of this phenomenon is an increased extracellular adenosine level due to a reduced adenosine transport mediated by the equilibrative nucleoside transporter 1 (hENT1). It is reported that hENT1 is reduced by transcriptional repression mediated by an increased nitric oxide (NO)-dependent activation of the human C/EBP homologous protein 10 (hCHOP). Moreover, in vitro studies have reported a beneficial role of insulin in the normalization of the GDM-induced hENT1 alterations. This phenomenon has been described in GDMd, but whether this hENT1 dysregulation takes place in GDMi remains to be studied. In this chapter, we have used human umbilical vein endothelial cells (HUVECs), as a representative of the fetoplacental endothelium, from normal (n=5), GDMd (n=6) and GDMi (n=6). HUVECs from the three groups were isolated by collagenase digestion and cultured with primary culture medium. Cells were incubated in the presence or absence of insulin (1 nmol/L, 8 hours). In these cells, we evaluated mRNA and protein levels of hENT1, with RT-qPCR and Western blot, respectively. In addition, protein levels of hCHOP were evaluated with Western blot. Like previous studies, we found a decreased hENT1 mRNA in HUVECs from GDMd compared to HUVECs from normal pregnancies. Similarly to GDMd, GDMi reduced hENT1 mRNA. Moreover, GDMd reduced the hENT1 protein abundance, a phenomenon abolished by insulin therapy. Furthermore, we found that in vitro insulin did not change the protein level of hENT1. Regarding hCHOP protein abundance, we did not find differences between the three groups. It is suggested that insulin therapy normalizes the GDM-associated hENT1 reduction, likely mediated by posttranslational mechanisms.

Introduction

Gestational diabetes mellitus (GDM) is a pregnancy disease characterized by D-glucose intolerance [1]. Women with GDM show hyperglycemia, a condition that is transferred to the fetal circulation [2]. The main goal of the treatment for women that develop GDM is the reduction of the glycemia to avoid the complications for the mother and the fetus [3–5]. For this, the first line of treatment is diet control (GDM*d*). However, the normalization of the glycemia to the recommended goals is not always achieved [4,6,7]. When this occurs, the pregnant women are subjected to insulin therapy (GDM*i*) [4–6,8]. Under both treatments, the GDM fetuses show a glycemia comparable to the maternal glycemia as in normal pregnancies [9]; however, the fetoplacental vascular alterations seen in the fetoplacental vasculature at birth to GDM pregnancies are maintained [9].

Fetoplacental vasculature from GDM pregnancies show alterations in the adenosine/L-arginine/nitric oxide (ALANO) pathway [10]. HUVECs from GDM*d* exhibit increased expression and activity of the endothelial nitric oxide synthase (eNOS) and human cationic amino acid transporter 1 (hCAT-1) with the subsequent increased nitric oxide (NO) synthesis [11–13]. Moreover, a decreased expression and activity of the human equilibrative nucleoside transporter 1 (hENT1) is seen in these cells [14,15], leading to an extracellular accumulation of adenosine [14,16]. Adenosine is an endogenous vasoactive nucleoside and in GDM HUVECs activates A2A adenosine receptors increasing the hCAT-1–mediated L-arginine transport and NO synthesis [17]. Interestingly, in GDM*d* activity of the human C/EBP homologous protein 10 (hCHOP) leads to the transcriptional repression of hENT1 in response to an increased NO level. Additionally, *in vitro* insulin normalizes the expression of hENT1 in HUVECs from GDM*d* pregnancies [14,18].

Alterations in the endothelial NO synthase (eNOS) and hCAT-1 expression and activity seen in GDM*d* are maintained HUVECs from GDM*i* [9]. However, the effect of insulin therapy on hENT1 expression is still unknown. In this study we evaluated the effect of insulin therapy in the expression of hENT1 and the potential involvement of hCHOP in this phenomenon in HUVECs from GDM pregnancies.

Materials and methods

Study groups

Samples were collected from full-term normal, GDM*d* and

GDM*i* pregnancies from the Hospital Clínico UC-CHRISTUS and Hospital San Juan de Dios in Santiago de Chile. Pregnant women did not smoke or consume drugs or alcohol and had no intrauterine infection or other medical or obstetrical complications. The investigation conforms to the principles outlined in the Declaration of Helsinki. Ethics Committee approvals from the Faculty of Medicine of the Pontificia Universidad Católica de Chile and HSJD, and informed written consent of patients were obtained. All the pregnant women were normoglycemic in the first trimester of pregnancy. Patients with basal glycemia ≥5.56 (≥100 mg/dL, 8-9 h from the last feeding in at least two different days) and with ≥7.9 mmol/L (≥140 mg/dL) at 2 h after glucose load at the second or third trimester of pregnancy were diagnosed with GDM (according to the Perinatal Guide 2015 report from the Health Ministry of Chile) [19]. Three groups of pregnant women were included, i.e. normal pregnancies, GDM*d* pregnancies, or GDM*i* pregnancies in which the mother was treated with insulin (i.e. on insulin therapy). Qualified personnel of the HCUC-C and HSJD selected the groups of pregnant women according to their clinical characteristics following established protocols. Women with normal pregnancies followed standard self-controlled diet and physical activity. Women with GDM*d* were subjected to dietary treatment with 1500 kcal/day and a maximum of 200 g per day carbohydrates. Plasma glucose was weekly measured at fasting and post-breakfast, before and 1 h after the midday and evening meals. Women with GDM*d* that did not reach normal glycemia after two weeks of dietary intervention were passed into insulin therapy following protocols adopted at the Division of Obstetrics and Gynecology at the HCUC-C and HSJD as recommended in the Perinatal Guide 2015 Chile [19]. In brief, women with GDM*d* not responsive to diet were treated for 8–10 weeks until delivery with two injections (one before breakfast and one at bedtime) of neutral protamine Hagerdon human insulin (Humulin-N or Insuman-N, 0.5–0.65 units/kg of pre-pregnancy body weight). Additional three injections of regular (rapid-acting) insulin (Humulin-R or Insuman-R) depending on pre-prandial self-controlled capillary glycemia (0.1 to 6 units/kg per meal for 81–120 to > 250 mg/dL glucose, respectively) were administered to women with GDM*i* as described for insulin therapy in pregnant women with pre-gestational diabetes mellitus type 1 or 2 [9,20–22]

Human placenta and umbilical cords

Placentas were collected at delivery on ice and transferred to the

laboratory until use 15–30 min later as described [9]. Middle sections of umbilical cords (100–120 mm length) were dissected into 200 mL phosphate-buffered saline (PBS) solution (mmol/L: 130 NaCl, 2.7 KCl, 0.8 Na2HPO4, 1.4 KH2PO4, pH 7.4, 4 °C) until use 6–12 h later for isolation of endothelial cells.

Cell culture and conditions

HUVECs were isolated by collagenase digestion (0.25 mg/mL collagenase) from umbilical cords obtained at birth from normal, GDM*d*, or GDM*i* pregnancies and cultured (37°C, 5% CO2) in 1% gelatin-coated petri dishes (60 mm diameter) up to passage 3 in primary culture medium (PCM; M199 containing 5 mmol/L D-glucose, 10% newborn calf serum (NBCS), 10% fetal calf serum (FCS), 3.2 mmol/L L-glutamine and 100 U/mL penicillin- streptomycin) [9]. Twelve hours prior experiments the incubation medium was changed to M199 medium containing 2% FCS. For some experiments, HUVECs were incubated (8 h) in the presence and the absence of insulin 1 nmol/L insulin (Humulin R, recombinant insulin) in M199 with 2% FCS.

RNA isolation and reverse transcription

HUVECs were washed (2 times) in cold (4°C) sterile PBS and total RNA was isolated using a Qiagen RNAeasy Kit (Qiagen, Venlo, The Netherlands) as described by the manufacturer. RNA concentration and purity were measured by spectrophotometric analysis (OD 260/280) and aliquots of 1 µg of total RNA were used for reverse transcriptase cDNA synthesis as described [9,23].

Quantitative RT-PCR (RT-qPCR)

Experiments were performed in a StepOn Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) in a reaction mix containing 0.2 µmol/L primers, 1 µL cDNA and 5 µL master mix (2 times) provided by Fast SYBR Green Master Mix (Applied Biosystems, Life Technologies). Taq DNA polymerase was activated (20 s, 95°C), denaturated (3 s, 95°C), and annealed and extended (30 s, 57 °C) for hENT1 mRNA and 28S rRNA (internal reference). At the end of the assay, a melting curve was performed. The primer efficiency was determined in a seriated dilution of PCR products specific for each gene obtained from agarose gel with Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). The oligonucleotide primers used were: hENT1 sense, 5′-TCTCCAACTCTCAGCCCACCAA-3′ and hENT1 antisense, 5′-

CCTGCGATGCTGGACTTGACCT-3 ′ ; 2 8S sense, 5 ′ -TTGAAAATCCGGGGGAGAG-3′ and 28S antisense, 5′-ACATTGTT CCAACATGCCAG-3′ [24].

Western blotting

Total protein was obtained from confluent cells rinsed (2 times) with ice-cold PBS and harvested in 100 µL of lysis buffer (63.7 mmol/ L Tris-HCl (pH 6.8), 10% glycerol, 2% sodium dodecylsulphate, 1 mmol/L sodium orthovanadate, 50 mg/mL leupeptin, 5% β-mercaptoethanol) as described [9,24]. Cells were sonicated (6 cycles, 5 s, 100 W, 4°C) and total protein was isolated by centrifugation (13500 g, 15 min, 4°C). Proteins (50 µg) were separated by polyacrylamide gel (10%) electrophoresis, transferred to Immobilon-P polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hertfordshire, UK) and probed (2 h, 22°C) with primary monoclonal rabbit anti-hENT1 (1:500) (catalogue number: M4200; Spring Bioscience, Pleasanton, CA, USA), hCHOP (GADD 153; catalogue number sc-71136, Santa Cruz Biotechnology) or anti-β-actin (1:5000, internal reference) (catalogue number: sc-8432; Santa Cruz Biotechnology) antibodies. The membranes were rinsed in Tris-buffered saline-0.1% Tween-20 (TBS-T) and further incubated (1 h) in TBS-T/0.2% bovine serum albumin containing secondary horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibodies (Thermo Scientific, Rockford, IL, USA). Proteins were detected by enhanced chemiluminescence ChemiDoc*i*t 510 Imagen System (UVP LCC, Upland, CA, USA) and quantified by densitometry using ImageJ (NIH) [9,24].

Statistical analysis

Data was analyzed with Graphpad prism 7a (San diego, CA, USA). Values for clinical parameters are expressed as mean ± S.D. Data for *in vitro* experiments are expressed as mean ± S.E.M. One-way or two-way analysis of variance (ANOVA) was used to compare three or more groups. In case of a significant one or two-way ANOVA, holm-sidak or sidak *post hoc* were respectively used. $p < 0.05$ was considered as statistically significant.

Results

Study groups.

Pregnant women showed, on average, similar age and height. They were normotensive and with normal fasting glycemia at term (Table 1). All the pregnancies included were singleton. The weight of

Table 1. Maternal and newborn characteristics.

Variables	Normal (n=5)	GDM*d* (n=6)	GDM*i* (n=6)
Maternal			
Age (years)	31.6 ± 3.4	34.5 ± 3.3	34.2 ± 3.5
Height (cm)	164.2 ± 5.4	159.6 ± 5.5	159.2 ± 3.5
Weight (kg)			
1st trimester	65.2 ± 9.5	71.2 ± 10.9	81.4 ± 18.2
2nd trimester	69.8 ± 8.8	74.4 ± 9.9	80.8 ± 17.7
3rd trimester	74.8 ± 6.3	76.3 ± 8.8	84 ± 15.2
BMI (kg/m²)			
1st trimester	24.1 ± 2.7	27.3 ± 3.7	32.3 ± 7.4
2nd trimester	25.8 ± 2.5	28.6 ± 3.6	32 ± 7.1
3rd trimester	27.7 ± 1	29.3 ± 3.2	33.2 ± 6
Systolic arterial pressure (mm Hg)			
1st trimester	108 ± 8.4	116.3 ± 10.2	96.7 ±5.8
2nd trimester	111 ± 20	112.3 ± 11.5	105 ± 10
3rd trimester	113.6 ± 5	106.5 ± 9.6	117.7 ± 9
Diastolic arterial pressure (mm Hg)			
1st trimester	63.6 ± 5.9	69.4 ± 8.3	63.3 ± 5.8 †
2nd trimester	70 ± 10	69.9 ± 8.7	64 ± 7.1
3rd trimester	69.6 ± 7.1	68.3 ± 7.2	70.4 ± 7.1
Fasting glycemia (mg/dL)	84.3 ± 11.9	84 ± 2.8	78.8 ± 12
OGTT (mg/dL) (2nd or 3rd trimester)			
Basal glycemia	86 ± 14.8	85.2 ± 10.2	81.4 ± 8.9
Glycemia after 2 h of glucose	111.2 ± 22.4	159.7 ± 18.7 *	180.8 ± 26.6 *
Newborn			
Sex (female/male)	6/5	8/6	9/6
Gestational age (weeks)	37.9 ± 1.2	38.8 ± 0.8	38.5 ± 0.4
Birth weight (grams)	3374 ± 215	3230 ± 283	3386 ± 645
Height (cm)	49.8 ± 1.3	49.9 ± 1.4	50.6 ± 1.5
Ponderal index (grams/cm³x100)	2.7 ± 0.1	2.6 ± 0.3	2.6 ± 0.4

Women that had normal pregnancies (Normal), GDM subjected to controlled diet (GDM*d*) or GDM*d* subjected to insulin therapy (GDM*i*) were included in this study. Maternal clinical characteristics were evaluated for 1st (0-14 weeks of gestation (wg)), 2nd (14-28 wg) and 3rd (28-40 wg) trimester. The body mass index (BMI) was calculated by weight in kilograms divided by the square of the height in meters. The ponderal index was calculated by weight in grams divided by the cube of height in centimeters multiplied by 100; OGTT, oral glucose tolerance test evaluated at 2nd or 3rd trimester depending the moment that GDM was diagnosed. Values are mean ± S.D. ∗ p-value < 0.05 vs. corresponding values in Normal. † p-value < 0.05 vs. corresponding values in GDM*d*.

women in the GDM*i* group was slightly higher at the third trimester, with the subsequent effect on the body mass index (BMI). However, no significant differences in these parameters were found. The clinical parameters of the newborns from the three study groups did not show differences (Table 1).

Insulin therapy does not affect GDMd-induced hENT1 mRNA.

HUVECs from GDM*d* or GDM*i* show reduced hENT1 mRNA expression compared with HUVECs from normal pregnancies (Fig. 1). Moreover, no differences were found in the hENT1 mRNA relative expression in cells from women with GDM treated with diet or insulin.

Insulin therapy restores hENT1 reduction in HUVECs from GDM.

We then evaluated the hENT1 protein abundance in HUVECs from normal and GDM pregnancies. Despite the fact that insulin therapy did not prevent the effect of GDM on the hENT1 mRNA relative expression, insulin therapy partially normalized the hENT1 protein abundance reduction observed in GDM*d* (Fig 2). Since insulin and NO are reported as a regulator of hENT1 expression, we also evaluated the effect of insulin *in vitro*. In HUVECs isolated from placentas obtained from normal, GDM*d* and GDM*i*, we did not find a significant effect of insulin (Fig 2).

GDM does not alter hCHOP total protein abundance.

Since hCHOP activity results in reduced expression of hENT1 in HUVECs from GDM, we here evaluated the protein abundance of hCHOP in HUVECs from normal, GDM*d* and GDM*i* pregnancies. Western blot analysis of hCHOP protein abundance in GDM*d* and GDM*i* did not show differences compared with the protein abundance in HUVECs from normal pregnancies (Fig. 3). Moreover,

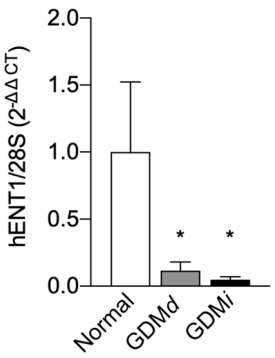

Figure 1. GDM is associated with lower hENT1 mRNA relative expression in HUVECs. The expression of the mRNA of hENT1 was determined in HUVECs from normal (Normal), GDM*d* and GDMi pregnancies. Groups were compared using one-way ANOVA and Holm-Sidak multiple comparison test. *p-value <0.05 vs. normal group. (n = 5 for normal, 6 for GDM*d*, and 6 for GDM*i*).

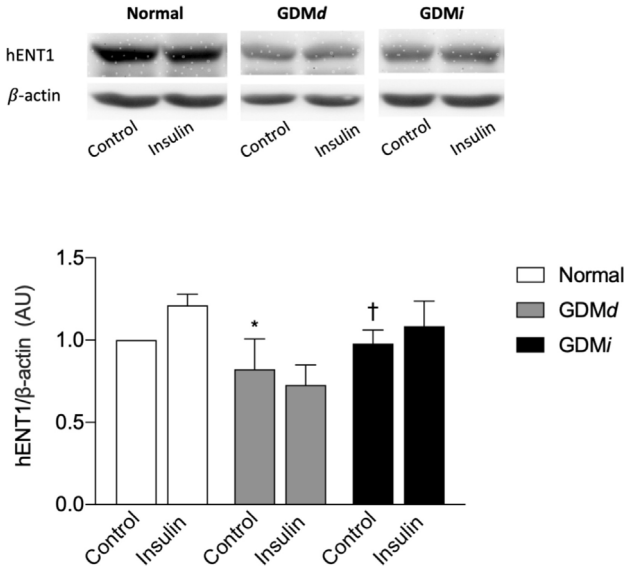

Figure 2. Insulin therapy partially restores the hENT1 protein abundance. hENT1 protein level was analyzed by Western blot (ß-actin as reference) in HUVECs isolated from normal, GDM*d* and GDM*i* pregnancies in the absence (Control) or presence of insulin (1 nmol/L, 8 h). Representative Western blot and densitometry analysis is shown. Groups were analyzed using two-way ANOVA (Sidak *post hoc*). * p-value 0.05 vs. normal group. † p-value <0.05 vs. GDM*d*. (n = 4 for normal, 4 for GDM*d*, and 4 for GDM*i*).

in vitro incubation with insulin (1 nmol/L) did not change the hCHOP protein abundance in the three groups.

Discussion

This study shows that insulin therapy has a potential effect on the prevention of the changes in one of the main regulators of adenosine metabolism, hENT1. Our results suggest that even though insulin therapy seems not to be enough to restore or prevent the effects of GDM regarding hENT1 mRNA in HUVECs, it prevents the reduction in the hENT1 protein abundance. In view of these results, it seems likely that a post-translational mechanism is triggered by insulin therapy in order to increase the protein level of hENT1. It is reported that the incubation of HUVECs from GDM*d* with insulin leads to a restored expression of hENT1 [14,18]. Therefore, in this study, we evaluated the effect of the incubation (i.e. *in* vitro) with insulin. However, the results did not show a significant effect of this

hormone in HUVECs, as has been described in other studies [14,18]. Moreover, we found that total protein abundance of hCHOP, a modulator of the transcriptional regulation of hENT1[15], is not altered in GDM*d* and GDM*i*.

GDM is detrimental for the fetoplacental vasculature [25–27]. Several studies have reported the effects in the endothelium [13–16,28–30]. One of the main alterations of this vascular bed is a dysregulation in the ALANO pathway [10,13]. Adenosine is increased in the placental circulation in GDM [14,16], this phenomenon results in activation of adenosine receptors leading to increased transport of L-arginine and expression and activation of eNOS. Increased L-arginine transport and eNOS activity lead to increased nitric oxide synthesis. It was recently reported that insulin therapy does not restore the alterations in the fetoplacental vasculature from GDM pregnancies, and an increased activation of eNOS, L-arginine transport and altered vasodilation are maintained [9]. However, in the present study HUVECs from GDM*i* showed a normalization in the protein abundance of hENT1. Nevertheless, in our study, the GDM*i* group had, on average, higher (but yet not significant) weight compared with normal and GDM*d*. This could represent a confounding factor since some of the alterations in the ALANO pathway have also been reported in other pregnancy conditions

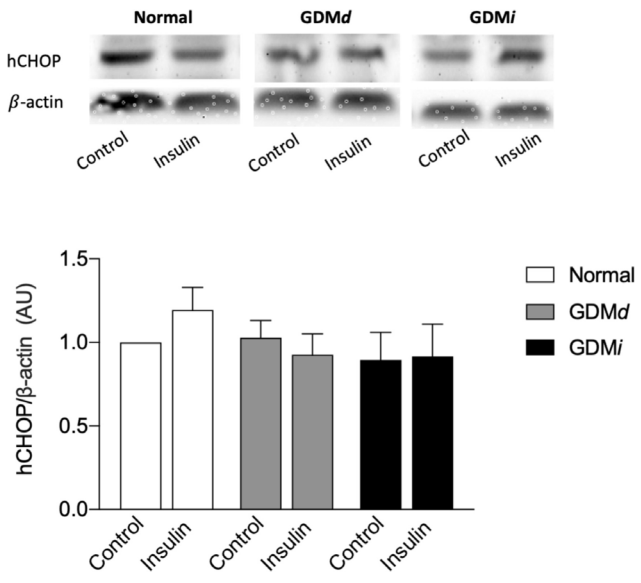

Figure 3. Total hCHOP protein abundance in HUVECs from GDM. hCHOP protein level was analyzed by Western blot (ß-actin as reference) in HUVECs isolated from normal, GDM *d* and GDM *i* pregnancies in the absence or presence of insulin (1 nmol/L, 8 h). Representative Western blot and densitometry analysis is shown. Groups were analyzed using two-way ANOVA. (n = 4 for normal, n = 4 for GDM*d*, and n = 4 for GDM*i*).

related to the maternal weight [23–25]. Therefore, our findings could show an effect of maternal weight and the GDM along with or instead of insulin therapy. More studies in a bigger group of GDM*i* stratified by weight should be performed in order to evaluate the independent effect of the GDM. However, the use of insulin therapy in GDM pregnancies is not uniform and different criteria are applied [5,6] making it difficult to obtain insulin-treated patients without other conditions. Additionally, maternal insulin does not cross the placenta, therefore the beneficial effect must be mediated by changes induced in molecules in the maternal circulation that can cross the placenta to induce an effect on the fetoplacental vasculature. In women with GDM*i* a reduction in triglycerides is reported [31]. This could be explained by an accumulation of lipids in the skeletal muscle, liver and other tissues induced by insulin therapy as reported in patients with type 2 diabetes mellitus [32,33]. Dyslipidemia in GDM has been proposed as a contributor for endothelial dysfunction in the fetoplacental vasculature [34], therefore a reduction in circulant lipids might imply lower lipid levels in the fetal circulation reducing the effects of GDM in this vascular bed. However, no information is available regarding lipid levels and changes in hENT1 and further studies are required to evaluate whether our data is explained by the reduction of triglycerides in GDM*i*.

The results show that the mRNA expression of hENT1 in HUVECs is similar in GDM*d* and GDM*i*. It is reported that NO is important in the regulation of the transcriptional activity of hENT1[12,15], leading to a transcriptional repression of *SCL29A1* (for hENT1) mediated by hCHOP in HUVECs from GDM*d* pregnancies. We found no differences in the total abundance of hCHOP in HUVECs from both GDM groups compared to cells from normal pregnancies. However, in this study, we did not analyze whether GDM*i* affects the nuclear localization and the binding of hCHOP to *SCL29A1* promoter. In HUVECs from GDM*i* the NO level, due to an increased activation of eNOS and hCAT-1 is similar to GDM*d* [9]; therefore, the mechanisms leading to a lower *SCL29A1* transcriptional activity are maintained in HUVECs from GDM*i*, likely explaining the lower mRNA expression of hENT1 observed in the endothelial cells from these pregnancies.

The description of post-translational mechanisms that regulate hENT1 protein stability is scarce. It is reported that PKC delta and epsilon can activate the hENT1-mediated transport, likely in a post-translational manner [35]. In HUVECs from GDM*i* pregnancies a higher expression of insulin receptor A is reported [9].

This phenomenon might increase the effect of fetal insulin, leading to higher expression of PKC epsilon, as it reported in rat adipocytes [36]. Increased PKC epsilon activity may explain the increased protein abundance of hENT1 in HUVECs from GDM*i*. Moreover, activation of A_1 adenosine receptors increases the hENT1 activity and trafficking in a PKC delta-dependent manner in PK15 cell line [37]. Interestingly, A_1 adenosine receptors are required to facilitate the insulin effect in the reversion of GDM-increased L-arginine transport [17], suggesting that the activation of this subtype of adenosine receptors might trigger beneficial cellular effects improving insulin effect counteracting the effect of GDM in the fetoplacental vasculature.

In summary, insulin therapy normalizes the protein expression of hENT1 in HUVECs from GDM pregnancies, likely in a post-translational dependent manner. This suggests that this treatment might exert a partial beneficial effect avoiding some of the alterations described in GDM fetoplacental vasculature. Nevertheless, further characterization of the functional parameters of hENT1 and hCHOP, and the mechanisms behind the insulin therapy-associated normalization of hENT1 protein abundance normalization is required. It is worth considering that the protocols for insulin therapy are diverse and other conditions such as obesity or excessive gestational weight gain can be present in mothers that require alternatives to GDM diet intervention. Additionally, the studies including GDM groups treated with insulin therapy is scarce and the beneficial and/or detrimental effect of this treatment is contradictory

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